# EXHIBIT 1

# *Molecular Cloning*

## A LABORATORY MANUAL

## SECOND EDITION

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## HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)

- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)

- Degree and method of agitation (continuous shaking or stationary)

- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix

- Concentration of the labeled probe and its specific activity

- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or poly-ethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids

- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.

2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.

3. Continual movement of the probe solution across the filter is un-necessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneous-ly, agitation is advisable to prevent the filters from adhering to one another.

4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are un-known. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 $\mu$g of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where $x$ = the weight of the probe added (in micrograms), $y$ = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and $z$ = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by pre-hybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 $\mu$g/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/ Hogness hybridization [1975], Benton/Davis hybridization [1977], South-ern hybridization [1975] of abundant DNA sequences, etc.), we recom-mend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfac-tory. In general, Denhardt's reagent is more effective for nylon mem-branes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

**TABLE 9.1  Blocking Agents Used to Suppress Background in Hybridization Experiments**

| Agent | Recommended uses |
| --- | --- |
| Denhardt's reagent | northern hybridizations<br>hybridizations using RNA probes<br>single-copy Southern hybridizations<br>hybridizations involving DNA immobilized<br>   on nylon membranes |

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and $H_2O$ to 500 ml.

| | |
| --- | --- |
| BLOTTO | Grunstein/Hogness hybridization<br>Benton/Davis hybridization<br>all Southern hybridizations other than<br>   single-copy<br>dot blots |

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

**Caution:** Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

| | |
| --- | --- |
| Heparin | Southern hybridization<br>in situ hybridization |

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

| | |
| --- | --- |
| Denatured, fragmented<br>  salmon sperm DNA | Southern and northern hybridizations |

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The $OD^{260}$ of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in hybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature ($T_m$). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated $T_m$ of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = $10^9$ cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = $10^9$ cpm/μg or greater).

11. *Useful facts*:

   a. The $T_m$ of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G} + \text{C}) - 0.63(\% \text{ formamide}) - (600/l)$$

where $l$ = the length of the hybrid in base pairs.
This equation is valid for:

- Concentrations of $\text{Na}^+$ in the range of 0.01 M to 0.4 M. It predicts $T_m$ less accurately in solutions of higher $[\text{Na}^+]$.

- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of $T_m$ in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" $T_m$ that is defined by optical measurement of hyperchromicity at $OD_{257}$. The "irreversible" $T_m$, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation. Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{G} + \text{C}) + 11.8(\% \text{G} + \text{C})^2 - 0.35(\% \text{ formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\% \text{G} + \text{C}) + 11.8(\% \text{G} + \text{C})^2 - 0.50(\% \text{ formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the $T_m$ of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the $T_m$ of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The $T_m$ of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

## Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

   *Prehybridization solutions*

   For detection of low-abundance sequences:

   > *Either*
   > 6 × SSC (or 6 × SSPE)
   > 5 × Denhardt's reagent
   > 0.5% SDS
   > 100 μg/ml denatured, fragmented salmon sperm DNA
   >
   > *or*
   > 6 × SSC (or 6 × SSPE)
   > 5 × Denhardt's reagent
   > 0.5% SDS
   > 100 μg/ml denatured, fragmented salmon sperm DNA
   > 50% formamide

   For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

   *Formamide:* Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   For detection of moderate- or high-abundance sequences:

   > *Either*
   > 6 × SSC (or 6 × SSPE)
   > 0.05 × BLOTTO
   >
   > *or*
   > 6 × SSC (or 6 × SSPE)
   > 0.05 × BLOTTO
   > 50% formamide

   For preparation of BLOTTO, see Table 9.1.

When $^{32}$P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 $\mu$g/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.
   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 $\mu$g of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10$^9$ cpm/$\mu$g or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10$^9$ cpm/$\mu$g or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

*Hybridization solution for nylon membranes*

> 6 × SSC (or 6 × SSPE)
> 0.5% SDS
> 100 μg/ml denatured, fragmented salmon sperm DNA
> 50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2 × SSC and 0.5% SDS at room temperature.

   **Important:** Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2 × SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1 × SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1 × SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

( > 2000 cps on a hand-held minimonitor), hot ( > 500 cps on a hand-held minimonitor), and cool ( > 50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

## Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and $\alpha_1$-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Geliebter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.

2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.

3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.

4. Oligonucleotides are radiolabeled by [$\gamma$-$^{32}$P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.

5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).

6. Wherever possible, negative and positive hybridization controls should be included in each gel.

7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 $\mu$g of total cellular RNA (Zeff et al. 1986) or 5 $\mu$g of poly(A)$^+$ RNA (Geliebter et al. 1986).

# EXHIBIT 2

CLUSTAL W (1.82) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: HrpW              1275 bp
Sequence 2: HrpZ              1038 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:   2
Guide tree        file created:    [/ebi/extserv/clustalw-work/interactive/clustalw-2
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1:                      Delayed
Sequence:2      Score:9937
Alignment Score 2686
CLUSTAL-Alignment file created   [/ebi/extserv/clustalw-work/interactive/clustalw-200

CLUSTAL W (1.82) multiple sequence alignment

```
HrpW     ATGAGCAT-CGGCATCACA-CCCCGGCCGCAACAGACCACCACGCCACTCGATTTTTCGG  58
HrpZ     ------ATGCAGAGTCTCAGTCTTAACAGCAGCACGCTGCAAAGCCCGTCGAT-----GG  49
                   **  *  *   **  **   *      *  *** **  *   *  *  *** *****      **

HrpW     CGCTAAGCGGCAAGAGTCCTCAACCAAACACGTTCGGCGAGCAGAACACTCAGCAAGCGA  118
HrpZ     CGCTCGTTCTGATCCGTCCTGAAACCGAGACAACCGGGTCG-AGTACATCCAGC---CGG  105
                   ****         *     ***** **   *   *  **       ***    *  **  ***    ****      **

HrpW     TCGACCCGAGTGCACTGTTGTTCGGCAGCGACACACAGAAAGACGTCAACTTCGGCACGC  178
HrpZ     GCGCTTCAGGAA----GTGATTGCGCAGCT-TGCCCAGGAGCTGACTCACAATGGTCAAC  160
                   **    *  *         **   **   *****    *  *** *          **    **        *

HrpW     CCGACAGCACCGTCCAGAATCCGCAGGACGCCAGCAAGCCCAACGACAGCCAGTCCAACA  238
HrpZ     TGGACGAGAGT-TCGCCATTGGGCAAGTTGCTCGGCAAAGCGATGGCCGCCAG-------  212
                     ***     *    **   *  *   *** * **    *   *     *  *  *  * *****

HrpW     TCGCTAAATTGATCAGTGCATTGATCATGTCGTTGCTGCAGATGCTCACCAACTCCAATA  298
HrpZ     TGGCAAGGCTGGCGGCGGCCTCGAGGACATCAAGGCTGC-GCTGGACACGCTTATCCACG  271
                   *  ** *    **      ** *  **   *   **    ***** *  **    ***       *  *

HrpW     AAAAGCAGGACACCAATCAGGAACAGCCTGATAGCCAGGCTCCTT-TCCAGAACAACGGC  357
HrpZ     AAAAGCTGGGCGACAATTTTGGTGCTTCTGCCGACAACGCCTCGGATACCGGACAACA-C  330
                   ****** **  *    ****    *       ***      *   *  **     *   *  *  * *****    *

HrpW     GGGCTCGGTACACCGTCGGCCGATAGCGGGGGCGGCGGTACACCGGATGCGACAGGTGGC  417
HrpZ     GACTTGATGACACAGGTGCTCAATGGCCTGGCCAAGTCGATGCTGAATG-ATCTTCTGAC  389
                   *      *      ****  *    *   *  **  **    ** *         *   *  *  ***      *       ** *

HrpW     GGCGGCGGTGATACGCCAAGCGCAACAGGCGGTGGCGGCGGTGATACTCCGA--CCGCAA  475
HrpZ     CA-AGCAG-GATG------ACGGAACTCGTTTTTTCCGAGGACGACATGCCGATGCTGAAA  441
                       **  *  ***          **  ***   *     *    **    *   **   *  ****    *   *   **

HrpW     CAGGCGGTGGCGGCAGCGG-TGGCGGCG--GCACACCCACTGCAACAGGTGGCGGCAGCG  532
HrpZ     AAGATCGCGGAGTTCATGGACGACAACCCCGCACAGTTTCCCAAACCGGACTCGGGTTCG  501
                    **    *  **  *      **    *  *  *    *****      *     *** **     ***    **

HrpW     GTGGCACACCCACTGCAACAGG----CGGTGGCGAGGGTGGCGTAACACCGCAAATC-AC  587
HrpZ     TGGGTGAACGAACTCAAGGAAGATAACTTCCTCGACGGCGACGAAACAGCGCAGTTCCGC  561
                    **     **    ***   *   *  *        *** **  *  **   **** ****     **   *

HrpW     TCCGCAGTTGGCCAACCCTAACCGTACCTCAGGTACTGGCTCGGTGTCGGACACCGCAGG  647
HrpZ     TCGGCACTCGAC-------ATCATCGGCCAGCAATTGGGCAGTCAACAGAA--TGCAG-  610
                   ** *** * * *         *  *    *** * ***    *    *  **    ****

HrpW     TTCTACCGAGCAAGCCGGCAAGATCAATG-TGGTGAA--AGACACCATCAAGGTCGGCGC  704
HrpZ     --CCGGCGGTCTGGCGGGCGATAGCAGCGGTGGTGGCTTGGGCAGCCCTGTGAGCAATAC  668
                      *     **  *   **  ***  *   **   *  *****       *   ** *       *    *       *

HrpW     TGGCGAAGTCTTTGACGGCCACGGCGCAACCTTCACTGCCGACAAATCTATGGGTAACGG  764
HrpZ     GG--AAAATTCGCCTGGTTCACTGGGTGATCCGC-TTATCGACGCTAACACCGGCCCCGC  725
                    *     ** *         *   *** *  *  *  *   *  *    ****       *    **     **

HrpW     AGACCAGGGCGAAAATCAGAAGCCCATGTTCGAGCTGGCTGAAGGCGCTACGTTGAAGAA  824
HrpZ     CAGCAACAGC--AATTCCAATGGTGATGT--GGGCCAGTTGA---TCGGTGAGCTGA----  775
                       *  *   **   ** **  * *    ****    *  **   * ***    ** *    * ***

HrpW     TGTGAACCTGGGTGAGAACGAGGTCGATGGCATCCACGTGAAAGCCAAAAACGCTCAGGA  884
HrpZ     --TAGACCGGGG----ACTGCAATCGGTATTGGCCGGTGGCGGCTTGGGAACACCTGTGA  829
                     *   *** ***        *    *   *** *        **     *       *** *      **
```

```
HrpW    AGTCACCATTGACAACGTGCATGCCCAGAACGTCGGTGAAGACCTGATTACGGTCAAAGG 944
HrpZ    G--CACCGCCAATACTGCCCTTGTGCC---TGGCGGCGAACAGCCGAATCAGGACCTGGG 884
            ****      *  *   *  * **   *      * *** *** * * ** *   ** *    **

HrpW    CGAGGGAGGCGCAGCGGTCACTAATCTGAACATCAAGAACAGCAGTGCCAAAGGTGCAGA 1004
HrpZ    TCAGTTA--CTCGGTGGCTTGTTGCAGAAAGGTCTGGAA--GCGACGCTTCAGG------ 934
            **   *   * * * **      *        ** ** *** **      **    ***

HrpW    CGACAAGGTTGTCCAGCTCAACGCCAACACTCACTTGAAAATCGAC--AACTTCAAGGCC 1062
HrpZ    -----ATGCTGGCCAGACCGGGACCGGCGTGCAGTCCAGCGCTGCTCAAGTTGCCTTGCT 989
              * * ** ****   *     ** *   ** *  *        *     *    *   **

HrpW    GACGATTTCGGCACGATGGTTCGCACCAACGGTGGCAAGCAGTTTGATGACATGAGCATC 1122
HrpZ    GCTGGT--CAACATGCTGCTGCAAAGCAC---TAAAAACCAGGCTGCTG-CCTGA----- 1038
        *   *  *   *   ** * ** *  *  * **    *    ** ***   ** **  * ***

HrpW    GAGCTGAACGGCATCGAAGCTAACCACGGCAAGTTCGCCCTGGTGAAAAGCGACAGTGAC 1182
HrpZ    ------------------------------------------------------------

HrpW    GATCTGAAGCTGGCAACGGGCAACATCGCCATGACCGACGTCAAACACGCCTACGATAAA 1242
HrpZ    ------------------------------------------------------------

HrpW    ACCCAGGCATCGACCCAACACACCGAGCTTTGA 1275
HrpZ    ---------------------------------
```

# EXHIBIT 3

```
CLUSTAL W (1.82) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: HrpW            1275 bp
Sequence 2: HrpN            1023 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:   4
Guide tree      file created:    [/ebi/extserv/clustalw-work/interactive/clustalw-2
Start of Multiple Alignment
There are 1 groups
Aligning...
Group 1:                     Delayed
Sequence:2      Score:9956
Alignment Score 2596
CLUSTAL-Alignment file created  [/ebi/extserv/clustalw-work/interactive/clustalw-200
```

```
CLUSTAL W (1.82) multiple sequence alignment


HrpW        ATGAGCATCGGCATCACACCCCGGCCGCAACAGACCACCACGCCACTCGATTTTTCGGCG 60
HrpN        ------------------------------------------------------------


HrpW        CTAAGCGGCAAGAGTCCTCAACCAAACACGTTCGGCGAGCAGAACACTCAGCAAGCGATC 120
HrpN        ------------------------------------------------------------


HrpW        GACCCGAGTGCACTGTTGTTCGGCAGCGACACACAGAAAGACGTCAACTTCGGCACGCCC 180
HrpN        ---------------------------------------------------------AT 2


HrpW        GACAGCACCGTCCAGAATCCGCAGGACGCCAGCAAGCCCAACGACAGCCAGTCCAACATC 240
HrpN        GCAAATTACGATCAAAG--CGCACATCGGCGGTGATTTGGGCGTCT-CCGGTCTGGGGCT 59
            *    *    **  ** *   ****   ** * *   * **   * ** ***

HrpW        GCTAAATTGATCAGTGCATTGATC--ATGTCGTTGCTGCA--GATGCTCACCAACTCCAA 296
HrpN        GGGTGCTCAGGGACTGAAAGGACTGAATTCCGCGGCTTCATCGCTGGGTTCCAGCGTGGA 119
            *     *     * ** *   **      **   **   *** **   * **   *** *         *

HrpW        TAAAAAGCAGGACACCAATCAGGAACAGCCTGATAGCCAGGCTCCTTTCCAGAACAACGG 356
HrpN        TAAACTGAGCAGCACCATC----GATAAGTTGACCTCCGCGCTGACTTCGATGATGTTTG 175
            ****  *     *****      * *   ***   **   ***   *** *  *       *

HrpW        CGGGCTCGGTACACCGTCGGCCGATAGCGGGGGCGGCGGTACACCGGATGCGACAGGTGG 416
HrpN        GCGGCGCGCTGGCGCAGGGGCTGGGCGCCAGCTCGAAGGGGCTGGGGATG-AGCAATCAA 234
             *** ** *     *   *** *   **   *   **   ** **  * *  *****   **

HrpW        CGGCGGCGGTGATACGCCAA--GCGCAACAGGCGGTGGCGGCGGTGATACTCCGACCGCA 474
HrpN        CTGGGCCAGTCTTTCGGCAATGGCGCGCAGGGTGCGAGCAACC-TGCTATCCGTACCGAA 293
            * * * * **    * ** *** ****   ** *   **  * ** **   *  **** *

HrpW        ACAGGCGGTGGCGGCAGCGGTGGCGGCGGCACACCCACTGCAACAGGTGGCGGCAGCGGT 534
HrpN        ATC--------CGGCGGCGATG-CGTTGTCAAAAATGTTTGATAAAGCGCTGG--ACGAT 342
            *          **** *** ** **   * ** *         *   *  * *   **  ** *

HrpW        GGCACACCCACTGCAACAGGCGGTGGCGAGGGTGGCGTAACACCGCAAATCACTCCGCAG 594
HrpN        ----CTGCTGGGTCATGACACCGTGACCAAGCTGACTAACCAGAGCAACCAACTGGCTAA 398
                *   *      **   *   * *** * * **  *    * **   **** ***    *

HrpW        TTGGCCAACCCTAACCGTACCTCAGGTACTGGCTCGGTGTCGGACACCGCAGGTTCTACC 654
HrpN        TT---CAATGCTGAACGCCAGCCAGAT---GACCCAGGGTAATAT------GAATGCGTT 446
            **    ***  ** * **     *** *   *  * * *  **   *       *   *

HrpW        GAGCAAGCCGGCAAGATCAATGTGGTGAAAGACACCATCAAGGTCGGCGCTGGCGAAGTC 714
HrpN        CGGCAG--CGGTGTGAACAACGCACTGTCGTCCATTCTCGGCAACGGTCTCGGCCA-GTC 503
              ***     ***   ** *** *   **     **   **   *** ***  * ***

HrpW        TTTGACGGCCACGGCGCAACCTTCACTGCCGACAAATCTATGGGTAACGGAGACCAGGGC 774
HrpN        GATGAGTGGCTTCTCTCAGCCTTCTCTGGGGGCAGGCGGCTTGCAGGGCCTGAGCGGCGC 563
            ***  * *     * ** ***** ***  * **       * *        ** * * **

HrpW        GAAAATCAGAAGCCCAT--GTTCGAGCTGGCTGAAGGCGCTACGTTGAAGAATGTGAACC 832
HrpN        GGGTGCATTCAACCAGTTGGGTAATGCCATCGGCATGGGC-GTGGGGCAGAATGCTGCGC 622
            *          * **  *  * *    **   * * * **   *  * ****** *

HrpW        TGGGTGAGAACGAGGTCGATGGCATCCACGTGAAAGCCAAAAACGCTCAGGAAGTCACCA 892
HrpN        TGAGTGCGTTGAGTAACGTCAGCACCCACGTAGACGGTAACAACCGCCA------CTTTG 676
            ** *** *       **   *** ****** * *   ** ***   **         *.
```

```
HrpW   TTGACAACGTGCATGCCCAGAACGTCGGTGAAGACCTGATTACGGTCAAAGGCGAGGGAG 952
HrpN   TAGATAAAGAAGAT-CGCGGCATGGCGAAAGAGATC-GGCCA-GTTTATGGATCAGTATC 733
       *  ** **  *     **  *   * *  *  **    *** * *    * * * *   *   **

HrpW   GCGCAGCGGTCACTAATCTGAACATCAAGAACAGCAGTGCCAAAGGTGCAGACGACAAGG 1012
HrpN   CGGAAATATTCGGTAAACCGGAATACCAGAAAGATGGC-TGGAGTTCGCCGAAGAC--GG 790
          *  *     **   *** *  *  *   * ****     *       *      ** **  ***    **

HrpW   TTGTCCAGCTCAACGCCAACACTCACTTGAAAATCGACAACTTCAAGGCCGACGATTTCG 1072
HrpN   ACGACAAATCCTGGGCTAAAGCGC---TGAG------------TAAACCGGATGATGACG 835
          *   *     *      **  **   *  *       ***                ** * ** ***   **

HrpW   GCACGATGGTTCGCACCAACGGTGGCAAGCAGTTTGATGACATGAGCATCGAGCTGAACG 1132
HrpN   GTATGACCG---GCGCCAGCATGGACAAATTCCGTCAGGCGATGGGTATGATCAAAAGCG 892
       *  *  **    *      **  *** *      *  ***       *  *  *   *** * **          *  **

HrpW   GCATCGAAGCTAACCACGGCAAGTTCGCCCTGGTGAAAAGCGACAGTGACGATCTGAAGC 1192
HrpN   CGGTGGCGGGTGATACCGGCAATACCAACCTGAACCTGCGTGGCGCGGGCGGTGCATCGC 952
          *  *    * *   *    ******     *    ****        * * *     *  ** *        **

HrpW   TGGCAACGGGCAACATCGCCATGACCGACGTCAAACACGCCTACGATAAAACCCAGGCAT 1252
HrpN   TGGGTAT---CGATGCGGCTGTCGTCGGCGATAAAATAGCCAAC-ATGTCGCTGGGTAAG 1008
       ***  *     * *     **  *    ** **   *** *** ** **       *    *   *

HrpW   CGACCCAACACACCGAGCTTTGA 1275
HrpN   CTGGCCAACGC-CTGA------- 1023
       *    ***** * *  **
```